Published in final edited form as:

Clin Chim Acta. 2010 October 9; 411(19-20): 1474–1481. doi:10.1016/j.cca.2010.05.046.

Performance Evaluation of three Liquid Chromatography Mass Spectrometry Methods for Broad Spectrum Drug Screening

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Abstract

BACKGROUND—Liquid chromatography-mass spectrometry (LC-MS) and tandem LC-MS (LC-MS/MS) are increasingly used in toxicology laboratories as a complementary method to gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-ultraviolet detection (LC-UV) for comprehensive drug screening (CDS). This study was designed to characterize the sensitivity and specificity of three LC-MS(/MS) vendor-supplied methods for targeted CDS and identify the current limitations associated with the use of these technologies.

METHODS—Five methods for broad spectrum CDS, including LC-UV (REMEDi), full scan GC-MS, LC-MS (ZQ™-Mass Detector with MassLynx™-software), LC-QTRAP-MS/MS (3200-QTRAP® with Cliquant®-software) and LC-LIT-MS/MS (LXQ™ Linear Ion Trap with ToxID™-software) were evaluated based on their ability to detect drugs in 48 patient urine samples.

RESULTS—The tandem MS methods identified 15% more drugs than the single stage MS or LC-UV methods. Use of two broad spectrum screening methods identified more drugs than any single system alone. False negatives and false positives generated by the LC-MS(/MS) software programs were identified upon manual review of the raw data.

CONCLUSIONS—The LC-MS/MS methods detected a broader menu of drugs; however, it is essential to establish manual data review criteria for all LC-MS(/MS) drug screening methods. Use of an EI-GC-MS and ESI-LC-MS/MS combination for targeted CDS may be optimal due to the complementary nature of the chromatographic and ionization techniques.

Keywords

Liquid Chromatography; Mass Spectrometry; General Unknown Screening; Comprehensive Drug Screening

Introduction

In many clinical laboratories, gas chromatography-mass spectrometry (GC-MS), liquid chromatography with ultraviolet detection (LC-UV) and/or thin-layer chromatography have

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been used for comprehensive drug screening (CDS), also referred to as general unknown screening. The limitations of LC-UV are well established, which include the limited specificity and variability of UV spectra. Also, many drugs have little to no UV absorbance, restricting the menu of detectable drugs. GC-MS has higher sensitivity and specificity than LC-UV, however GC-MS is not capable of directly analyzing drugs that are non-volatile, polar or thermally labile [1]. In addition, lengthy sample preparations, which include hydrolysis and derivatization, are required prior to GC-MS analysis. Despite these limitations, GC-MS is still considered the gold standard technique for CDS in the toxicology laboratory due to its widespread availability, high specificity and high reproducibility of generated mass spectra using electron ionization (EI). Large transferable EI-mass spectra libraries exist and are available for library searching of acquired spectra from any instrument [2].

Recently, some clinical toxicology laboratories have adopted liquid chromatography-mass spectrometry (LC-MS) as a complementary method to GC-MS and LC-UV for CDS [3–6]. Unlike GC-MS, LC-MS(/MS) using electrospray ionization (ESI) is capable of detecting non-volatile, polar and thermally labile drugs and provides a means of detecting a broad menu of drugs without the need for lengthy sample preparations. Because urine is the specimen of choice for CDS, LC is also ideal because drugs and metabolites are already in a polar environment. ESI is a soft ionization process and thus fragmentation must be produced by in-source collision-induced dissociation (CID) for single MS systems or CID in the second quadrupole, for tandem MS systems. Product ion spectra produced by CID differ markedly between instruments and within instruments depending on instrument settings (e.g. collision energy) [7]. This poses a problem for the creation of large product ion spectra libraries utilized by chromatographic data analysis software for broad spectrum drug screening. Despite attempts to standardize the production of the product ion spectra [8–11], ion relative intensities are highly variable requiring the need for the development of search algorithms that do not rely on relative intensities but rather on the absence or presence of ions.

Methods for broad spectrum drug screening have been established for a variety of LC-MS/MS systems. Most recently developed methods are considered multi-target screening procedures rather than comprehensive drug screens because they screen for a defined number of drugs using selected reaction monitoring (SRM). SRM is accomplished by specifying the parent mass of the drug for MS/MS fragmentation and then specifically monitoring for a single fragment ion. Multi-targeted methods often use SRM as the initial survey scan. The most abundant ions in this scan are identified and selectively fragmented in the collision cell by CID. The resulting ions are separated according to m/z and a full scan product ion spectrum is recorded. Some tandem MS instruments perform these various scans in time within one quadrupole, referred to as a linear-ion trap (LC-LIT), while others perform these scans in space utilizing the first quadrupole for the survey scan, the second for CID (collision cell) and the third as a regular quadrupole or a linear ion trap for the analysis of the product ions. Recent work suggests that the reproducibility of the product ion spectra is increased when comparing tandem in time MS/MS instruments and tandem in space MS/MS instruments [12].

Instrument-specific product ion spectra libraries have been created [12–17] and vendors are now marketing pre-developed broad spectrum drug screening methods, with data analysis software for library searching and drug identification. There are many barriers that the average clinical toxicology laboratory has to overcome in order to implement these technologies into routine clinical practice, including a limited supply of technologists with mass spectrometry expertise, inadequate funding for expensive instrumentation, difficulties with method optimization and lengthy data analysis processes. Guidelines for qualitative

analytical toxicology analysis have been published by several organizations [18–21]; however, no consensus guidelines have been established [22,23]. Therefore, clinical toxicology laboratories are left to individually determine the criteria necessary for positive identification of a drug adding to the complexity of the data analysis process.

For many years the Bio-Rad REMEDi drug profiling system was utilized in laboratories as a commercial black-box solution for CDS, however Bio-Rad made the decision to discontinue supporting this system at the end of 2008. This study was designed to characterize one LC-MS and two LC-MS/MS commercial methods that were developed by vendors as a potential solution for broad spectrum drug screening in the clinical laboratory and to evaluate their complementary relationship to traditional LC-UV (REMEDi) and GC-MS methods. Our goal was to implement the vendor recommendations and determine if these methods could serve as a turn-key replacement for the REMEDi system. Furthermore, we outline the analytical limitations associated with the use of these technologies in the clinical laboratory.

Materials and Methods

STANDARDS AND REAGENTS

Organic solvents and reagents were of analytical grade. Acetonitrile, water, methanol, formic acid, ammonium formate, sodium acetate, acetic acid, borate buffer, dichloromethane, chloroform, hexane, isoamyl alcohol, ethyl acetate and acetic anhydride were purchased from Fisher Scientific (Fair Lawn, NJ) or Sigma-Aldrich (St. Louis, MO). Hydrochloric acid, sodium hydroxide, ammonium sulfate, 2-propanol and pyridine were purchased from VWR International (West Chester, PA). Human drug free urine was purchased from UTAK Laboratories, Inc. (Valencia, CA). Drug standards and labeled internal standards were purchased from Cerilliant (Round Rock, TX), Grace Davison/Alltech (Deerfield, IL), Sigma-Aldrich (St. Louis, MO) and Lipomed Inc. (Cambridge, MA).

DRUG SCREENING METHODS

Key features of all five methodologies are summarized in Table 1.

REMEDi method (LC-UV)—The REMEDi method was performed as previously described on the Bio-Rad REMEDi Drug Profiling System [24]. Briefly, the samples were prepared by mixing 1 mL urine and 200 μ L of internal standard mix (N-ethyl-nordiazepam and chorpheniramine). The samples were vortex mixed and centrifuged for 5 min at 10,000 rpm and then extracted online using two polymeric cartridges (purification and extraction columns) and separated using two analytical cartridges (separation I and II cartridges). Separation was carried out under isocratic conditions and the analysis time was 20 minutes. Drug identification was performed by a multiwave length full-scan UV detector, scanning from 200 nm to 300 nm, coupled with a computer algorithm. Sample spectra were compared with the library of known drug spectra. This technology is no longer supported by Bio-Rad.

GC-MS method—Samples were analyzed according to the method described in [2]. The samples were prepared by mixing 2 mL urine with 0.8 mL 6 M hydrochloric acid and incubated at 110°C for 20 minutes for hydrolysis. After cooling, urine samples were adjusted to pH 8–9 with 1.2 mL of 10 M sodium hydroxide and 1.6 mL 2.3 M ammonium sulfate. The pH was checked with indicator paper. Subsequently another 2 mL of neat urine was added and 4 mL of extraction solvent (ethylacetate/dichloromethane/2-propanol, 3:1:1 v/v/v). The samples were mixed for 10 minutes and centrifuged for 20 minutes (2000 rpm). The organic layer was transferred into a new screw capped tube and evaporated under air stream at room temperature. To the dry residue 80 μ L of freshly prepared derivatization

reagent (acetic anhydride/pyridine 3:2 v/v) was added and derivatization was performed with microwave irradiation (400W, 5 minutes). After evaporation of excess derivatizing reagent, the residue was reconstituted in 100 μ L methanol and 1 μ L was injected onto an HP-1 Methyl Siloxane Capillary Column (12 m $\times$ 0.2 mm $\times$ 0.33 μ m) (Agilent Cat# 19091-60312). If less than 4 mL sample was available, the urine volume was equally split between hydrolysis and neat, while the difference in volume was made up with deionized water.

The samples were analyzed on a 6890N Network GC System (Agilent) with 7683 B series injector (Agilent) and 5975 inert XL Mass Selective Detector (Agilent) operated by HP Enhanced Chemstation Software (MSD ChemStation D02.00.275 1989–2005). The GC conditions were: splitless injector mode with injection port at 280°C; carrier gas helium at flow rate of 1.0 mL/min; column temperature initially at 100°C for 3 minutes, programmed to increase at 30°C/min to 310°C and final time 5 minutes, total run time was 15 minutes. The conditions for the mass spectrometer for the screening procedure were: full-scan mode (m/z 50–450 Da) with a threshold at 150 counts, scan rate 3.58 scans/sec, electron ionization mode, ionization energy 70 eV, ion source temperature 230°C.

The GC-MS system acquired data files that were reviewed for identification of peaks and spectra of drugs as their parent and/or metabolite(s) in derivatized or underivatized form using the PMW library [2] and an In-House library of additional drugs. Identification was obtained by computer assisted comparison of mass spectra for all relevant peaks with those of the PMW library and our In-House library.

LC-ZQ-MS method—The samples were prepared by mixing 1 mL of urine, 200 μ L internal standard (Chlorpheniramine), 3 mL dichloromethane/chloroform/hexane 30/50/20 (v/v) with 0.5% isoamyl alcohol and either 500 μ L of 1 M sodium acetate, pH 3.5 or 500 μ L of 2.62 M borate, pH 9.5. The samples were mixed for 2 minutes and centrifuged for 5 minutes at 3000 rpm. The aqueous layers were removed and the organic layers were pooled, mixed and dried under nitrogen gas at 35°C. The samples were reconstituted with 200 μ L mobile phase A (see below). For LC, an Alliance 2695 separations module was used with a Waters XTerra MS C18, 3.5- μ m (100 $\times$ 2.1 mm) column, maintained at 30°C and a gradient of mobile phase A (5 mmol/L ammonium formate, pH 3.0) and mobile phase B (5 mmol/L ammonium formate in acetonitrile, pH 3.0). The program was 0–2 min, 5% B; 2–16 min, 5%–90% B; 16–26 min, equilibrate with 5% B. Detection was carried out with an ZQTM Single Quadrupole Mass Spectrometer controlled by MassLynxTM software (Waters Corporation, Milford, MA). Positive and negative ionization was performed with the following MS settings; capillary voltage, 3.5 kV; source temperature, 120°C; desolvation temperature, 250°C; desolvation gas flow rate, 350 l/h; and cone gas flow rate, 100 l/h. The method contained 7 functions and utilized in-source collision induced dissociation (CID). Function 1 was a negative ESI full scan from 100 to 650 amu in 250 ms at 30 volts. Functions 2 to 7 were positive ESI full scans from 100 to 650 amu in 250 ms from 15 volts to 90 volts. The generated spectra were compared against a library of 514 drugs which also contained retention times for each analyte. This data processing was done by ChromaLynxTM software using a unique search algorithm.

LC-QTRAP-MS/MS method—Urine samples were centrifuged at 2000 rpm for 10 minutes. The sample was prepared by mixing 100 μ L urine, 50 μ L internal standard (promazine, 100 μ g/mL) and 850 μ L of 10 mM ammonium formate, pH 3.0. For LC, an Agilent 1200 series was used with a Waters XTerra MS C18, 3.5- μ m (100 $\times$ 2.1 mm) column, maintained at 25°C and a gradient of mobile phase A (0.5 mmol/L ammonium formate, pH 3.0) and mobile phase B (acetonitrile:10 mmol/L ammonium formate, pH 3.0; 90:10 by volume). The program was 0–11 min, 5%–100% B, 300 μ L/min; 11–13 min, 100%

B, 300 $\mu\text{L}/\text{min}$; 13–15 min, 100%–5% B, 350 $\mu\text{L}/\text{min}$; 15–19 min, equilibration with 5% B, 350 $\mu\text{L}/\text{min}$. Detection was carried out with an Applied Biosystems QTRAP[®] LC-MS/MS system equipped with a TurboIon Spray[™] ionization source, controlled by Cliquant[®] 1.0 Drug Screen and Quant software with Analyst 1.4.2 software (Life Technologies/Applied Biosystems, Foster City, CA). Positive ionization was performed with the following settings: ion spray voltage, 4000V; curtain gas, 20 psi; ion source gas 1, 40 psi; ion source gas 2, 55 psi; CAD gas, high; declustering potential, 40V; and temperature, 500°C. The method included an initial multiple reaction monitoring (MRM) scan followed by an enhanced product ion (EPI) scan when the conditions of the criteria established in the Information Dependent Acquisition (IDA) were met. An EPI spectrum records the full range of product ions using Q3 as a linear ion trap. The resulting EPI spectrum was evaluated by the Cliquant[®] software to determine if there was a suitable match in the library. The MS survey scan contained 264 SRM transitions for drugs and/or metabolites. Each transition was performed with a 5 ms dwell time and fragmentation was performed in the collision cell (second quadrupole, Q2) with 3 different collision energies (35 ± 15 V). The IDA criteria were set to select for the 2 most intense peaks which exceed 1000 cps and exclude former target ions for 15 seconds after 4 occurrences. The mass tolerance was set at 0.25 amu. For the EPI scan the scan rate was set to 4000 amu/sec, Q0 trapping was on and the LIT fill time was set to 50 ms.

LC-LIT-MS/MS method—The samples were prepared by mixing 1 mL of urine, 100 μL internal standard mix (prazepam-D5 and haloperidol-D4, 100 $\mu\text{g}/\text{mL}$) and 2 mL of 0.1 M phosphate buffered drug free urine, pH 6. After vortex mixing, the samples were loaded onto an SPE column (Hypersep Verify-CX 200 mg mixed mode cartridges, Thermo Fisher Scientific), previously conditioned with 2 mL methanol and 2 mL phosphate buffer, pH 6. After rinsing the cartridge with 1 mL water, 0.5 mL 0.01 M acetic acid and 50 μL methanol, elution of the acidic and neutral fractions was performed with 1.5 mL acetone/chloroform 50/50 (v/v) and 1.5 mL acetone/dichloromethane 50/50 (v/v). The basic fraction was then eluted with 1.5 mL ethyl acetate/ammonium hydroxide 98/2 (v/v) and 1.5 mL dichloromethane/isopropanol/ammonium hydroxide 78/20/2 (v/v/v). Extracts were evaporated dry and reconstituted in 100 μL deionized water/acetonitrile 1/1 (v/v) and 0.1% formic acid. For LC, a Surveyor MS Pump Plus was used with a Thermo Scientific Hypersil GOLD PFP, 5- μm (150 $\times$ 2.1 mm) column and a gradient of mobile phase A (10 mmol/L ammonium formate, 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid). The flow rate was 200 $\mu\text{L}/\text{min}$ with a gradient program as follows: 0–5 min, 5%–45% B; 5–18 min, 45%–70% B; 18–20 min, 70%–95% B; 20–25 min, 95% B; 25–30 min, equilibration with 5% B. Detection was carried out with a Thermo LXQ[™] Linear Ion Trap mass spectrometer equipped with a ESI, Ion Max[™] Source and reports were generated using ToxID[™] software (Thermo Fisher Scientific, San Jose, CA). Positive and negative ionization was performed with the following MS settings; capillary temperature, 275°C; spray voltage, 5 kV; sheath gas, 30; auxiliary gas, 8; and step collision energy, $35\% \pm 10\%$. The data was acquired using a polarity switching scan dependent experiment. The first scan was a full MS scan in positive polarity which selected for masses present on a list of parent masses of interest. The five most abundant masses were then monitored in the next 5 MS/MS scans. The seventh scan was a full MS scan in negative polarity which selected for masses present on a list of parent masses of interest. This scan was followed by 1 MS/MS scan which monitored the most abundant mass present in the negative polarity full MS scan.

Method comparison approach—Forty-eight patient samples acquired from the clinical laboratories at San Francisco General Hospital and Johns Hopkins medical center were run on all five methods. These were left over samples from routine drug of abuse testing. The medical records for these patient samples were reviewed at the time of sample analysis and

the information obtained regarding prescribed medications was used in the interpretation of the analytical results. This study was reviewed and approved by the Institutional Review Boards at University of California San Francisco and The Johns Hopkins University, who determined that individual patient consent was unnecessary. Prior to running patient samples for the study, all methods were independently validated by the vendors and/or clinical laboratory in which they were operated. The number of drugs detected with each method was highly dependent upon which drugs and the total number of drugs each method was capable of detecting. The LC-ZQ-MS utilized a full scan method; therefore information was potentially acquired for all drugs present. However, only drugs present in the library searched by the software could be identified by automated data review. Both the LC-QTRAP-MS/MS and LC-LIT-MS/MS utilized a targeted method for identification, therefore, identification was dependent upon the presence of a drug in the method as well as the library. For the LC-MS(/MS) methods, the detectable drug menus included 514 (LC-MS), 264 (LC-QTRAP-MS/MS) and 277 (LC-LIT-MS/MS) drugs. There were only 53 drugs (see below) that were present in the detectable drug menus for all five methods; therefore the methods were also evaluated based on their ability to detect this subset of 53 drugs in the 48 patient samples.

Results

Evaluation of screening methods for detection of drugs in patient samples

The total number of drugs identified by each method, after manual data review, in the 48 patient samples were 123 (REMEDi), 206 (GC-MS), 146 (LC-ZQ-MS), 196 (LC-QTRAP-MS/MS) and 240 (LC-LIT-MS/MS). These numbers correspond to the detection of 60 (REMEDi), 75 (GC-MS), 58 (LC-ZQ-MS), 72 (LC-QTRAP-MS/MS) and 81 (LC-LIT-MS/MS) different drugs. The total number of drugs identified, using the common subset of drugs detected by each method (53 drugs—described above and listed in Table 3), after manual data review were 78 (REMEDi), 131 (GC-MS), 107 (LC-ZQ-MS), 126 (LC-QTRAP-MS/MS) and 151 (LC-LIT-MS/MS) (Table 2). Each drug in a patient sample counted as one. For example, if each of the 48 specimens contained 2 drugs from the subset of 53 drugs, then the total number of drugs identified by the method would be 96. Table 2 lists the number of expected drugs that were detected, the expected drugs that were not detected and the unexpected drugs that were detected. A drug was determined to be “expected” in a patient sample if, 1) the immunoassay for the drug and/or metabolite was positive, 2) the patient’s medical record listed a prescription for the drug at the time the sample was collected and 3) two or more of the drug screening methods were positive for the drug. For the drugs identified by each method 73 (REMEDi), 124 (GC-MS), 94 (LC-ZQ-MS), 122 (LC-QTRAP-MS/MS) and 130 (LC-LIT-MS/MS) were expected drugs, whereas 5 (REMEDi), 7 (GC-MS), 13 (LC-ZQ-MS), 4 (LC-QTRAP-MS/MS) and 21 (LC-LIT-MS/MS) were unexpected drugs and could potentially be false positives (Table 2). The number of expected drugs that were not detected was 83 (REMEDi), 32 (GC-MS), 62 (LC-ZQ-MS), 34 (LC-QTRAPMS/MS) and 26 (LC-LIT-MS/MS) (Table 2). Table 3 lists the subset of 53 drugs, the number of times the drug was expected to be positive in the patient samples and the number of times each was detected by the 5 methods. There were 48 drugs that were only detected by the LC-MS/MS methods (23% of all positives), compared to 8 drugs (4%) for the GC-MS and 1 drug for the REMEDi. There were 31 drugs (15% of all positives) that were only detected by the dual stage MS methods (LC-QTRAP-MS/MS and LC-LIT-MS/MS).

Comparison of LC-UV and GC-MS methods to LC-MS(/MS)

The percent of drugs that each LC-MS(/MS) method identified out of all the drugs identified by REMEDi or GC-MS was determined in order to compare the ability of the LC-MS(/MS)

methods to replace and/or complement these methods. Out of the total number of drugs detected by REMEDI, in the subset of 53, the LC-MS(/MS) methods detected 75.6% (LC-ZQ-MS), 89.7% (LC-QTRAP-MS/MS) and 93.6% (LC-LIT-MS/MS) (Table 4). There were no expected drugs that only the REMEDI was capable of detecting. Out of the 131 drugs detected by the GC-MS method the LC-MS(/MS) methods detected 58.3% (LC-ZQ-MS), 76.5% (LC-QTRAP-MS/MS) and 78.0% (LC-LIT-MS/MS) (Table 4). Since the GC-MS method included hydrolysis, lorazepam and other glucuronidated drugs such as morphine were detected with the GC-MS method in more samples (Table 3). The LC-MS/MS methods detected a higher percentage of drugs identified by REMEDI and GC-MS than the LC-MS method. Since only the vendor-recommended methods were used for the LC-MS(/MS) systems, no comparison was made between these methods due to the discrepancies between procedures (ie: differences in sample preparations, liquid chromatography and automated library matching criteria).

Significance of manual data review for all LC-MS(/MS) drug screening methods

The dual stage MS methods (LC-QTRAP-MS/MS and LC-LIT-MS/MS) may detect a broad menu of drugs and require less time for sample preparation compared to GC-MS; however, one of the main limitations of the LC-MS/MS methods was misidentification of drugs with the data analysis software's library search algorithms [26]. Therefore, it was necessary to establish data review criteria for each method to eliminate false positives and to find drugs missed by the search algorithms. The data review criteria established in this study for the LC-MS(/MS) methods are listed in the Supplemental Data Appendix 1. In short, three of the common criteria used for positive identification were, 1) relative retention time (RRt) from the internal standard was ≤ 0.2 from the expected RRt, 2) the acquired spectra matched the library spectra with a "high" score (there was a value specific to each method) and 3) there were at least three matching ions between the acquired spectra and library spectra. The other method specific criteria are outlined in Appendix 1. Out of the total number of drugs identified before data review 37.4% (LC-ZQ-MS), 36.4% (LC-QTRAP-MS/MS) and 35.7% (LC-LIT-MS/MS) were identified as false positives upon manual data review (Table 4). A common cause of these false positives was carryover (58.7% of the false positives for LC-LIT-MS/MS and 4.5% for LC-QTRAP-MS/MS). For all methods, the results were reviewed in the order that they were acquired. Samples were re-injected if they contained a drug that was present with a "large" peak area in the previous sample. The peak area threshold used to determine if a peak was "large" was method specific. To address the LC-LIT-MS/MS method carryover issue, an extra wash was added between each sample. Another common cause of false positives was nonspecific matching of sparse spectra (73.1% of false positives for LC-QTRAP-MS/MS and 16.8% of false positives for LC-LIT-MS/MS). These include nonspecific matching of sparse acquired spectra (<3 ions) to library spectra and nonspecific matching of acquired spectra to sparse library spectra (<3 ions).

Examples of library searching that resulted in the false identification of drugs are given in Fig. 1. The fit value (Fit %) reflects the similarity of the ions in the library spectra with those in the acquired spectra. The reverse fit (RFit %) corresponds to the similarity of the signals in the acquired spectra with those in the library spectra. The purity fit (Purity %) is a combination of the Fit and RFit values. A purity of $>70\%$ was used as the cutoff for a positive identification. This purity cut-off for positive identification was suggested by the manufacturer and was accepted for routine use after evaluation of over 50 clinical samples in which the drugs present were known. In 5 out of the 48 samples lamotrigine was positively identified by the search algorithm for the LC-QTRAP-MS/MS method. However, upon manual spectral review the acquired spectra for all five only contained one ion with m/z of 256.3. This ion matched to the lamotrigine library spectra with a Purity of $>70\%$ (Fig. 1A). Because there was only one ion in the acquired spectra and the RRt from the internal

standard was > 0.2 from the expected RRT, this did not meet the manual review criteria for positive identification and was considered a false positive in all 5 samples. The same scenario was true for other drugs with both LC-MS/MS methods. Similarly, amphetamine was considered a false positive in multiple samples for both LC-MS/MS methods (Fig. 1B). The acquired spectra contained one ion (m/z of 91) and the library spectra only contained 2 ions resulting in a high % purity. In some instances the relative retention time was ≤ 0.2 from the expected RRT. Amiodarone is an example of a drug for which a sparse library spectrum (1 ion) resulted in the false identification of acquired spectra (Fig. 1C). Such spectra should not be included in the product ion spectra libraries, however, multiple <3 ion spectra existed in both LC-MS/MS libraries.

Out of the total number of drugs identified with the LC-QTRAP-MS/MS and the LC-LIT-MS/MS after data review, 49.3% and 4.8% respectively, were not identified by the search algorithms but rather upon manual data review (Table 4). The common drugs that were missed did not correlate to retention time or molecular weight. There were 6 samples in which benzoylecgonine was present and was not correctly identified by the LC-QTRAP-MS/MS library search algorithm. In these samples, the peak area for benzoylecgonine was greater than $3e^6$ and resulted in the acquisition of a “corrupt spectra” due to an overloading of the linear ion trap and space-charge effects (Fig. 1D) [27]. If the peak was manually inspected a different product ion spectra could be found towards the end of the peak that when searched against the library resulted in a purity match of $>70\%$. The same space-charging effects were seen for other drugs and metabolites that were found at very high concentrations in patient samples. This is something that has to be considered when using a hybrid ion trap instrument, such as the LC-QTRAP-MS/MS.

Discussion

Only one study to date evaluates multiple methods, including LC-UV, GC-MS and LC-MS, for targeted CDS [28]. They concluded that LC-MS for CDS is complementary to LC-UV and GC-MS and enlarges the range of drugs detected in clinical toxicology [27]. Since then multiple LC-MS/MS CDS methods have been published some of which are commercially available for use in the clinical toxicology laboratory [16,17,28–31]. These methods have not been compared head-to-head with LC-UV and GC-MS methods, using clinical samples with multiple drugs. The current study evaluates one LC-MS and two LC-MS/MS commercially available broad spectrum screening methods and determines their complementary relationship to LC-UV and GC-MS methods. Similar to the study by Saint-Marcoux et al. we found that 23% of all drugs identified were obtained by LC-MS(/MS) alone [28]. Additionally, the dual stage MS methods together identified $> 15\%$ more drugs than the single stage MS or LC-UV techniques. Use of two broad spectrum screening methods identified more drugs than any single system alone. Based on these results, an EI-GC-MS and ESI-LC-MS/MS combination may be optimal due to the complementary nature of the chromatographic and ionization techniques.

It is important to note the limitations associated with these method comparisons. The number of drugs identified by the LC-MS(/MS) methods (Table 2 and 3) could be due in part to the differences in extraction methods. The LC-MS(/MS) sample extraction methods used in this study were the recommended methods developed by the vendors and were the methods being utilized in the respective labs in which these methods were run. As a result, the methods did not utilize the same extraction procedure. For example, the LC-LIT-MS/MS detected more drugs than the LC-QTRAP-MS/MS, but this could be because the sample preparation for the LC-LIT-MS/MS method concentrated the urine whereas the sample preparation for the LC-QTRAP-MS/MS diluted the urine. Determination of the optimal extraction method for targeted CDS using LC-MS(/MS) was beyond the scope of this paper.

The optimal extraction method may vary depending on the detection method used. Decisions relating to sample preparation should be addressed based on the time constraints and sensitivity needs of individual laboratories. Another limitation is that the GC-MS method utilized a library that was developed in-house as well as a commercially available library, while the LC-MS(/MS) methods only utilized a commercially developed library.

The results from this study indicate that caution should be taken if LC-MS/MS is used in isolation for CDS due to misidentification of drugs with the commercial available data analysis software's library search algorithms (Table 4). The libraries can be customized and the parameter settings can be altered to decrease or increase the threshold for collection of MS/MS spectral data. In this study, 35.7% of the drugs detected by the LC-LIT-MS/MS were determined to be false positives upon manual data review and 49.3% of the drugs detected by the LC-QTRAP-MS/MS were false negatives that were identified upon manual inspection. The LC-LIT-MS/MS method parameters for MS/MS spectral information acquisition and the library searching threshold were set in such a way to insure that drugs present were not missed; however this resulted in a high percentage of false positives. The opposite approach was used with the LC-QTRAP-MS/MS method. The parameters for MS/MS spectral information acquisition and the library searching threshold were set in attempts to eliminate false positives. In turn, this resulted in a high percentage of false negatives. Some of these false negatives were also due to a poor match between the library spectrum and the acquired spectrum. This can be addressed by adding a spectrum acquired on your own instrument to the library for automated searching or developing a library generated in-house. Future studies will need to be done in order to determine the parameter settings that are optimal for data collection in order to decrease the number of false positives and false negatives. Changing the parameters for spectra collection and adding spectra to the libraries are possible with each system, however, at this time there is no mechanism by which to change the default search criteria to reflect the established manual review criteria. Guidelines for positive identification and data review criteria will have to be established and the software programs will need to reflect these criteria.

Guidelines for qualitative clinical and forensic toxicology analysis have been published by several organizations [18–21]. These guidelines suggest that confirmation should be based on chromatographic retention (GC or LC) coupled with mass spectrometry (MS or MS/MS). Targeted methods are preferred over full scan methods. In the current study the LC-MS/MS targeted methods detected a broader menu of drugs compared to the LC-MS full scan method, in support of this guideline. Also, the guidelines state that two to four ions in the product ion spectra should be monitored, with a signal-to-noise ratio >3. This guideline is not automatically applied to the acquired data by the current library search algorithms. For this study, each acquired product ion spectrum had to be compared to the library spectrum manually to eliminate the software automated matches based on <3 ions (Fig. 1).

Despite the similarities just mentioned, the established guidelines differ significantly in their criteria for accepting the matching of the unknown mass spectrum with that of the reference standard. The primary focus of the established guidelines is for EI-GC-MS, however, additional considerations are necessary when using LC-MS(/MS) in order to overcome the low degree of intra and inter-instrument reproducibility. Some guidelines allow for the use of the library search algorithms to match spectra, but they remain very vague in defining what constitutes a satisfactory match. To complicate matters, the commercially available library search algorithms differ significantly and may give different answers. Most guidelines allow for the use of professional judgment when interpreting the data which can result in bias. The data presented in Table 4 and Fig. 1 strongly suggests that consensus guidelines for spectra review are necessary. We found that with the search algorithms used in this study, it is still essential to establish manual data review criteria for all drug screening

methods. For this study, the library search results of 50+ samples, containing known drugs, were analyzed in order to come up with review criteria for each individual LC-MS(/MS) method prior to analyzing clinical samples. These criteria were instrument and method specific (Supplemental Data Appendix 1). The numerous variables in the analytical methods and inconsistency among spectra produced by different LC-MS/MS instruments makes the development of consensus guidelines for positive identification technically difficult. Once consensus guidelines are established these will need to be added into automated data processing to decrease the lengthy data review procedures for the clinical laboratory.

These evaluations were conducted on urine samples submitted for clinical toxicology purposes. However, these methods and instrumentation are also valuable for forensic testing, especially toxicologists practicing in medical examiner offices where broad spectrum drug screening is important in determining the cause of death. Sample preparation procedures will have to be modified to accommodate the other types of postmortem samples that require testing, e.g., blood, vitreous, oral fluids, hair, liver tissues, etc.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported in part by the HIV Prevention Trials Network (HPTN) sponsored by the National Institute of Allergy and Infectious Diseases (NIAID), National Institute on Drug Abuse (NIDA), National Institute of Mental Health (NIMH), and Office of AIDS Research, of the NIH, DHHS (U01-AI-068613).

Abbreviations

LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
GC-MS	gas chromatography-mass spectrometry
LC-UV	liquid chromatography-ultraviolet detection
EI	electron ionization
ESI	electrospray ionization
GUS	general unknown screening
CID	collision-induced dissociation
LIT	linear ion trap
EME	ecgonine methyl ester
SRM	selected reaction monitoring
MRM	multiple reaction monitoring
IDA	information dependent acquisition
EPI	enhanced product ion

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Fig. 1. Examples of library searching that resulted in the false identification of drugs prior to manual data review

A) False positive for lamotrigine and B) amphetamine where acquired mass spectra containing 1 ion matched library spectra with >70% purity (cutoff for positive identification) (C) False positive for amiodarone where the library mass spectrum only contained 1 ion and matched the acquired mass spectrum from the sample with 94.1% purity (D) False negative for benzoyllecgonine where the acquired spectrum matched the library spectrum with <70% purity, due to space charging effects which resulted in a corrupt spectrum.

Table 1

Broad Spectrum Drug Screening Method Characteristics

	REMEDI	GC-MS	LC-ZQ-MS	LC-QTRAP-MS/MS	LC-LIT-MS/MS
Sample Preparation	online extraction	LLE	LLE	Dilute and Shoot	SPE
Separation Instrumentation	REMEDI Drug Profiling System (Bio-Rad)	6890N Network GC System (Agilent)	Alliance 2695 (Waters)	Agilent 1200 (Agilent)	Surveyor MS Pump Plus (Thermo)
Separation Column	cation exchange C18 (Bio-Rad)	0.33µm × 0.2mm × 12m HP-1 (Agilent)	3.5µm × 2.1mm × 150mm C18 (Xterra, Waters)	3.5µm × 2.1mm × 150mm C18 (Xterra, Waters)	5µm × 2.1mm × 150mm PPP (Hypersil GOLD, Thermo)
Mobile Phase	proprietary	n/a	A: 5 mmol/L NH ₄ HCO ₂ , pH 3 B: 5 mmol/L NH ₄ HCO ₂ in ACN	A: 0.5 mmol/L NH ₄ HCO ₂ , pH 3 B: 0.5 mmol/L NH ₄ HCO ₂ in ACN	A: 10 mmol/L NH ₄ HCO ₂ , 0.1% HCOOH B: ACN, 0.1% HCOOH
Gradient conditions	isocratic	100°C, hold 3 min, 100 to 310°C at 30°C/min, hold 5 min	0–2 min, 5% B 2–16 min, 5%–90% B 16–26 min, equilibrate 5% B	0–11 min, 5%–100% B 11–13 min, 100% B 13–15 min, 100%–5% B 15–19 min, equilibration 5% B	0–5 min, 5%–45% B 5–18 min, 45%–70% B 18–20 min, 70%–95% B 20–25 min, 95% B 25–30 min, equilibration 5% B
Detector Model	REMEDI Drug Profiling System (Bio-Rad)	5975 inert XL Mass Selective Detector (Agilent)	ZQ TM Mass Spectrometer (Waters)	3200 QTRAP® (Applied Biosystems)	LXQ TM Linear Ion Trap (Thermo)
Source	n/a	El (70 eV) scanning from 50 to 450 m/z	ESI (+/–)	Turbo Ion Spray TM Source ESI (+)	Ion Max TM Source ESI (+/–)
Mass Analyzer	n/a	single Quad (Q)	single Quad (Q)	Triple Quad LIT (QqLIT)	Linear Ion Trap (LIT)
Acquisition mode	scanning UV (200–300 nm)	full scan	in source CID/full scan	MRM-IDA-EPI	full scan targeted MS/MS
Instrument run time (min)	20	15	26	19	30
MS parameters	n/a	injector temperature: 280°C interface temperature: 280°C source temperature: 230°C quad temperature: 150°C	capillary voltage: 3.5 kV source temperature: 120°C desolvation temperature: 250°C desolvation gas flow rate: 350 l/h cone gas flow rate: 100 l/h	ion spray voltage: 4000V curtain gas: 20 psi ion source gas 1, 2: 40 and 55 psi source temperature: 500°C collision energy: 35 ± 10V	capillary temperature: 275°C spray voltage: 5 kV sheath and auxiliary gas: 30 and 8 step collision energy: 35% ± 10%
Analysis Software	n/a	Chemstation PMW Mass Spectral Library	ChromaLynx TM (Waters)	CliquiD® Drug Screen and Quant (Applied Biosystems)	ToxID TM (Thermo)

Table 2

Drugs Detected by each method

	Expected Drugs Detected	Expected Drugs not Detected	Unexpected Drugs Detected	Total Drugs Detected
REMEDi	73	83	5	78
GC-MS	124	32	7	131
LC-ZQ-MS	94	62	13	107
LC-QTRAP-MS/MS	122	34	4	126
LC-LIT-MS/MS	130	26	21	151

Table 3

Drugs detected by each method

Drug	Expected Positives*	REMEDI	GC-MS	LC-ZQ-MS	LC-QTRAP-MS/MS	LC-LIT-MS/MS
6-acetylmorphine	2	1	0	1	2	0
Acetaminophen	5	0	5	3	1	4
Amitriptyline	2	2	2	2	2	2
Amphetamine	1	0	1	0	0	1
Atenolol	2	0	0	0	2	2
Carbamazepine	3	1	3	3	3	3
Carisoprodol**	3	0	3	0	3	2
Chlorpheniramine	3	0	2	3	2	3
Citalopram	7	4	6	4	7	6
Clenbuterol	0	1	0	0	0	0
Clonazepam***	3	0	0	0	2	3
Clonidine	2	0	0	2	0	0
Clozapine	3	3	4	2	3	4
Cocaine [†]	12	9	7	12	11	17
Codeine	3	3	4	3	3	4
Dextromethorphan	4	0	4	2	3	4
Diphenhydramine	5	3	5	3	5	5
Disopyramide	0	1	0	0	0	0
Doxylamine	2	2	2	2	2	2
Ephedrine	2	2	2	2	2	0
Fentanyl	4	0	3	3	3	5
Fluoxetine	1	1	1	0	0	0
Gabapentin	9	0	7	0	8	7
Glipizide	1	0	0	1	1	1
Haloperidol	1	0	0	0	1	1
Hydroxyzine	2	0	2	2	2	3
Ketamine	1	0	1	1	1	1
Labetalol	2	2	3	1	2	6

Drug	Expected Positives*	REMEDi	GC-MS	LC-ZQ-MS	LC-QTRAP-MS/MS	LC-LIT-MS/MS
Lamotrigine	1	0	1	1	1	0
Lidocaine	4	3	2	2	4	5
Lorazepam	3	0	3	0	0	0
Meperidine	1	1	1	1	1	1
Methadone [‡]	8	7	8	16	8	11
Methamphetamine	2	2	1	0	1	2
Metoclopramide	2	1	1	2	2	2
Metoprolol	4	1	3	3	4	4
Mexilitene	1	1	1	1	1	1
Midazolam	2	0	1	1	0	2
Mirtazapine	1	0	1	1	1	1
Morphine	12	6	12	9	8	7
Nortriptyline	4	4	5	2	4	4
Oxazepam	2	0	2	0	1	0
Oxycodone	8	5	8	5	8	8
Paroxetine	2	1	3	2	1	2
Promethazine	4	3	4	1	0	4
Propranolol	1	1	0	1	1	1
Quinidine/Quinine	2	2	1	2	1	2
Setraline	1	1	1	0	1	1
Temazepam	1	0	1	1	1	1
Trinethoprim	2	2	2	0	2	3
Venlafaxine	1	1	1	0	1	1
Verapamil	1	1	2	1	1	1
Zolpidem	1	0	0	1	2	1

* Number of drugs expected to be positive based on medical records and drug screen results (see text for details)

** Carisoprodol and/or Meprobamate

*** Clonazepam and/or 7-aminoclonazepam

[‡] Cocaine, Benzoyllecgonine and/or Ecgoninemethylester

[†] Methadone and/or EDDP

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Table 4

Detection of False Positives and False Negatives upon manual data review

Analyzer	# drugs (before)	# drugs (after)	% false positive [†]	% false negative [‡]
LC-ZQ-MS	217	146	37.4	10.3
LC-QTRAP-MS/MS	146	196	36.4	49.3
LC-LIT-MS/MS	390	240	35.7	4.8

[†] drugs that did not meet the manual review criteria for positive identification

[‡] drugs not detected by the search algorithms but identified upon manual review